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(54) Title: ATP-BINDING CASSETTE TRANSPORTER (ABC1) MODIFIED TRANSGENIC MICE

(57) Abstract: A transgenic mouse with alterations in an *abc1* gene is prepared by introduction of an altered *abc1* gene into a host animal. The resulting transgenic mice do not produce functional ABC1 protein molecules. Cells and cell lines derived from these animals also contain the altered *abc1* gene.

ATP-BINDING CASSETTE TRANSPORTER (ABC1) MODIFIED TRANSGENIC MICE

FIELD OF THE INVENTION

The present invention relates to transgenic nonhuman animals wherein an *abc1* gene is altered, producing an animal lacking functional ATP-binding cassette transporter (ABC1) protein.

BACKGROUND OF THE INVENTION

The ABC superfamily is comprised of myriad transmembrane proteins involved in the transport of vitamins, peptides, ions, sugars and amino acids (Higgins, C.F. ABC-transporters: from microorgansisms to man. *Annu. Rev. Cell Biol.* 8, 67-113 (1992)).. A subset of the superfamily contains four closely related proteins (ABC1, ABC2, ABC3 and ABCR) (Luciani, M.F., Denizot, F., Savar, S., Mattei, M.G. & Chimini, G. Cloning of two novel ABC transporters mapping on human chromosome 9. *Genomics* 21, 150-159 (1994). Connors, T.D. *et al.* The cloning of a human ABC gene (ABC3) mapping to chromosome 16p13.3. *Genomics* 39, 231-234 (1997). Allikmets, R. *et al.* A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. *Nature Genet.* 15, 236-246 (1997)). The protein structure of this subfamily consists of two halves joined by a linker region, each with six transmembrane domains and an ATP-binding cassette site. Several diseases are the result of a dysfunctional ABC transporter - cystic fibrosis, Zellweger syndrome, adrenoleukodystrophy, multidrug resistance and Stargardt macular dystrophy - though the underlying molecular mechanism of the disease is seldom known (Allikmets, R. *et al.* A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. *Nature Genet.* 15, 236-246 (1997). Riordan, J.R. *et al.* Identification

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Mutations in the 70k peroxisomal membrane-protein gene in Zellweger syndrome. *Nature Genet.* 1, 16-23 (1992). Mosser, J. *et al.* Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. *Nature* 361, 726-730 (1993). Gottesman, M.M. & Patan, I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* 62, 385-428).

The relationship between TD (Tangier disease), FHA (familial hypoalphalipoproteinemia) and mutation in the human ABC1 gene has recently beenestablished by positional cloning of *hABC1* from several different families bearing TD or FHA (Bodzioch, M. *et al.* The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nature Genet.* 22, 347-351 (1999). Brooks-Wilson, A. *et al.* Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nature Genet.* 22, 336-345. Rust, S. *et al.* Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nature Genet.* 22, 352-355 (1999)). In each case, point mutations, deletions or frameshifts creates a non-functional or truncated hABC1 protein. Individuals suffer from reduced or absent HDL-C and lower serum cholesterol and may exhibit discolored tonsils, enlarged spleen and lymph nodes, corneal clouding and neuropathy due to deposition of cholesteryl esters. In other cholesterol metabolic diseases such as Apo A-I deficiency, LCAT deficiency and Fish Eye disease, HDL-C is also reduced but serum cholesterol levels remain normal (Assmann, G., von Eckardstein, A.. & Brewer, H.B. Jr Familial high density lipoprotein deficiency: Tangier disease. in *The Metabolic and Molecular Basis of Inherited Disease* (eds Scriver, C.R. *et al.*) 2053-2072 (McGraw-Hill, New York, 1995)). Low levels of HDL-C usually

indicate a high-risk factor for coronary heart disease. Interestingly, the loss of HDL-C in TD patients does not generally cause predisposition to coronary heart disease.

Tangier disease is exceedingly rare, with only 40 reported families worldwide exhibiting the trait. As such, except for the absence of HDL-C, it is difficult to evaluate which symptoms may be directly attributed to a dysfunctional ABC1 transporter. The murine model of Tangier disease, which is described here, confirms the association of ABC1 with TD and exhibits other phenotypes which have not yet been described in humans. The model may further elucidate why the loss of HDL-C does not directly cause coronary heart disease in TD patients.

The human ATP-binding cassette transporter 1 (hABC1), a member of the ABC superfamily, has recently been cloned and analyzed. Frameshift mutations and single base-pair deletions resulting in truncation of hABC1 have been described and linked to familial HDL deficiency (familial hypoalphalipoproteinemia or FHA) and Tangier disease (TD). Both diseases are characterized by the lowering or lack of HDL-C. Low serum cholesterol, splenomegaly, enlarged lymph nodes and the deposition of cholesteryl esters in the reticuloendothelial system are also associated with TD.

SUMMARY OF THE INVENTION

To understand the functional role of ABC1 in different cell types, mice that do not express the functional ABC1 were generated by homologous recombination (HR) in embryonic stem (ES) cells. These mice provide a valuable animal model and tools to understand the function of ABC1 and to

evaluate the therapeutic effects of drugs that modulate the function or the expression of ABC1 equivalents in human cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Construction and verification of ABC1 knockout. A) Disruption of the *abc1* gene. Genomic DNA fragment containing exons 18-22 of the *abc1* gene is shown. Exons 19, 20 and part of 21 that encode most of the first ATP binding cassette of the ABC1 protein were replaced with a neomycin resistance gene. Arrows denote PCR primers used to verify genotype. Small gray bar denotes hybridization probe used in Southern blot analysis. E = EcoRI, H = HinDIII B) Agarose gel of PCR amplified bands: lane 1-2 homozygote, lane 3-4 heterozygote, lane 5-6 wild-type. B) Southern blot analysis of EcoRI digest of genomic DNA. Lane 1 wild-type, lane 2 and 3 heterozygote (mouse lines 55.2 and 64.7).

Fig. 2. In-situ hybridization of ABC1 mRNA lining maternal decidual blood vessel derived from placenta, magnification 250x.

Fig. 3. Placenta malformation and oxidative distress in ABC1 knockout mice. [Panel 1] A) Day 14 placenta labyrinth from ABC1 knockout mouse line 64.7 stained with CD71, knockout (top) and wild-type (bottom), magnification 100x. [Panel 2] B) H&E stained day 14 placenta labyrinth knockout (top) and wild-type (bottom), magnification 63x. [Panel 3] C) Day 14 p.c. ABC1 knockout embryos demonstrating gradation of intrauterine growth retardation. [Panel 4] D) Day 19 embryos with amniotic sac, knockout (top) and wild-type (bottom). [Panel 5] E) H&E stained lungs from one-day-old neonates, knockout (top) and wild-type (bottom), magnification 100x.

Fig. 4. Kidney glomeruli nephritis and immunoglobulin deposition. [Panel 1] A) H&E stained kidney sections from diseased ABC1 knockout mouse line 55.2

(top) and healthy wild-type (bottom). [Panel 2] B) Heavy deposition of immunoglobulin in glomeruli of ABC1 knockout mouse line 64.7 (top) and Normal glomeruli (bottom).

DETAILED DESCRIPTION OF THE INVENTION

The ABC1 knockout mice were generated in the present invention by disruption of the *abc1* gene by homologous recombination (HR). The process of generating the knockout mice can be divided into 4 basic stages:

1. cloning of *abc1* gene and preparation of DNA construct for transfection of embryonic stem (ES) cells;
2. isolating ES cells in which the *abc1* gene has been disrupted by HR;
3. generating chimeric mice from mouse embryos injected with the knockout ES cells; and
4. breeding chimeric mice to obtain knockout mice through germline transmission.

The present invention utilizes a cloned genomic DNA encoding the ABC1 protein and describes the cloning and characterization of the mouse *abc1* gene. Transgenic animals are generated which have an altered *abc1* gene. The alterations to the naturally occurring gene can be modifications, deletions and substitutions. Modifications and deletions render the naturally occurring gene nonfunctional, producing a "knockout" animal. Substitution of the naturally occurring gene for a gene from a second species results in an animal which produces the gene product of the second species. Substitution of the naturally occurring gene for a gene having a mutation results in an animal which produces the mutated gene product. These transgenic animals are critical for drug antagonist or agonist studies, the creation of animal models of human diseases, and for eventual treatment of disorders or diseases associated with

ABC1-mediated responses. A transgenic animal carrying a "knockout" of ABC1 is useful for the establishment of a nonhuman model for diseases involving ABC1 equivalents in the human.

A transgenic mouse carrying the disrupted *abc1* gene was generated by homologous recombination of a target DNA construct with the endogenous gene in the chromosome. The DNA construct was prepared from a genomic clone of ABC1 that was isolated from a genomic DNA library.

The term "animal" is used herein to include all vertebrate animals except humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages. A "transgenic animal" is any animal containing one or more cells bearing genetic information altered or received, directly or indirectly, by deliberate genetic manipulation at a subcellular level, such as by targeted recombination or microinjection or infection with recombinant virus. The term "transgenic animal" is not intended to encompass classical cross-breeding or in vitro fertilization, but rather is meant to encompass animals in which one or more cells are altered by, or receive, a recombinant DNA molecule. This recombinant DNA molecule may be specifically targeted to a defined genetic locus, may be randomly integrated within a chromosome, or it may be extrachromosomally replicating DNA. The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic alteration or genetic information was introduced into a germ line cell, thereby conferring the ability to transfer the genetic information to offspring. If such offspring in fact possess some or all of that alteration or genetic information, they are transgenic animals as well.

The alteration or genetic information may be foreign to the species of animal to which the recipient belongs, or foreign only to the particular individual recipient, or may be genetic information already possessed by the recipient. In the last case, the altered or introduced gene may be expressed differently than the native gene, or not expressed at all.

The altered *abc1* gene generally should not fully encode the same ABC1 as native to the host animal, and its expression product should be altered to a minor or great degree, or absent altogether. However, it is conceivable that a more modestly modified *abc1* gene will fall within the scope of the present invention.

The genes used for altering a target gene may be obtained by a wide variety of techniques that include, but are not limited to, isolation from genomic sources, preparation of cDNAs from isolated mRNA templates, direct synthesis, or a combination thereof.

A type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells may be obtained from pre-implantation embryos cultured in vitro [M. J. Evans et al., Nature 292: 154-156 (1981); M. O. Bradley et al., Nature 309: 255-258 (1984); Gossler et al. Proc. Natl. Acad. Sci. USA 83: 9065-9069 (1986); Robertson et al., Nature 322, 445-448 (1986); S. A. Wood et al. Proc. Natl. Acad. Sci. USA 90: 4582-4584 (1993)]. Transgenes can be efficiently introduced into the ES cells by standard techniques such as DNA transfection or by retrovirus-mediated transduction. The resultant transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The introduced ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal (R. Jaenisch, Science 240: 1468-1474 (1988)).

Since ABC1 is an independent component of a complex mechanism, the proteins, including that encoded by *abc1* DNA, must be examined both individually and as a group if their contribution to the mechanisms are to be understood. One approach to the problem of determining the contributions of individual genes and their expression products is to use isolated genes to selectively inactivate the native wild-type gene in totipotent ES cells (such as those described herein) and then generate transgenic mice. The use of gene-targeted ES cells in the generation of gene-targeted transgenic mice was described in 1987 (Thomas et al., Cell 51:503-512, (1987)) and is reviewed elsewhere (Frohman et al., Cell 56:145-147 (1989); Capecchi, Trends in Genet. 5:70-76 (1989); Baribault et al., Mol. Biol. Med. 6:481-492, (1989); Wagner, EMBO J. 9: 3025-3032 (1990); Bradley et al., Bio/Technology 10: 534-539 (1992)).

Techniques are available to inactivate or alter any genetic region to any mutation desired by using targeted homologous recombination to insert specific changes into chromosomal genes. Homologous recombination was reported to be detected at frequencies between 10^{-6} and 10^{-3} (Lin et al., Proc. Natl. Acad. Sci. USA 82:1391-1395 (1985); Smithies et al., Nature 317: 230-234 (1985); Thomas et al., Cell 44:419-428, (1986); Song et al., Proc. Natl. Acad. Sci. USA 84:6820-6824 (1987)). Nonhomologous plasmid-chromosome interactions are more frequent, occurring at levels 10^5-fold (Lin et al., Proc. Natl. Acad. Sci. USA 82:1391-1395 (1985)) to 10^2-fold (Thomas et al., Cell 44:419-428 (1986); Song et al., Proc. Natl. Acad. Sci. USA 84:6820-6824 (1987)) greater than comparable homologous insertion.

To overcome this low proportion of targeted recombination in murine ES cells, various strategies have been developed to detect or select rare

homologous recombinants. One approach for detecting homologous alteration events uses the polymerase chain reaction (PCR) to screen pools of transformant cells for homologous insertion, followed by screening individual clones (Kim et al., Nucleic Acids Res. 16:8887-8903 (1988); Kim et al., Gene 103:227-233 (1991)). Alternatively, a positive genetic selection approach has been developed in which a marker gene is constructed which will only be active if homologous insertion occurs, allowing these recombinants to be selected directly (Sedivy et al., Proc. Natl. Acad. Sci. USA 86:227-231 (1989)). One of the most powerful approaches developed for selecting homologous recombinants is the positive-negative selection (PNS) method developed for genes (such as ABC1) for which no direct selection of the alteration exists (Mansour et al., Nature 336:348-352: (1988); Capecchi, Science 244:1288-1292, (1989); Capecchi, Trends in Genet. 5:70-76 (1989)). The PNS method is more efficient for targeting genes which are not expressed at high levels because the marker gene has its own promoter. Nonhomologous recombinants are selected against by using the Herpes Simplex virus thymidine kinase (HSV-TK) gene flanking the DNA construct. Cells with nonhomologous insertion of the construct express HSV thymidine kinase and therefore are sensitive to the herpes drugs such as ganciclovir (GANC) or FIAU (1-(2-deoxy 2-fluoro-B-D-arabinofluranosyl)-5-iodouracil). By this counter-selection, the number of homologous recombinants in the surviving transformants can be increased.

As used herein, a "targeted gene" or "knockout" is a DNA sequence introduced into the germline of a non-human animal by way of human intervention, including but not limited to, the methods described herein. The targeted genes of the invention include DNA sequences which are designed to specifically alter cognate endogenous genes.

All the above applications have to be verified in animal tests and eventually clinical trials. One approach to determine the functional role of the drug target is to study the defects resulting from the disrupted gene in a whole animal. The ABC1 knockout mice that have been generated and are disclosed herein will allow the definition of the function of ABC1, which is critical in deciding the types of modulators are most suitable in therapies.

Any ABC1 function that is detected in the knockout mice of the present invention would provide evidence of the existence of alternative novel ABC1 subtypes which may then be isolated from the knockout mice of the present invention.

The absence of functional ABC1 in the knockout mice of the present invention isconfirmed, for example, in RNA analysis, protein expression detection, and other ABC1 functional studies. For RNA analysis, RNA samples are prepared from macrophages harvested from the knockout mice and the ABC1 transcript was detected in Northern blots using DNA probes specific for the transcript.

The ABC superfamily is comprised of myriad transmembrane proteins involved in the transport of vitamins, peptides, ions, sugars and amino acids (Higgins, C.F. ABC-transporters: from microorgansisms to man. *Annu. Rev. Cell Biol.* 8, 67-113 (1992)).. A subset of the superfamily contains four closely related proteins (ABC1, ABC2, ABC3 and ABCR) (Luciani, M.F., Denizot, F., Savar, S., Mattei, M.G. & Chimini, G. Cloning of two novel ABC transporters mapping on human chromosome 9. *Genomics* 21, 150-159 (1994). Connors, T.D. *et al.* The cloning of a human ABC gene (ABC3) mapping to chromosome 16p13.3. *Genomics* 39, 231-234 (1997). Allikmets, R. *et al.* A photoreceptor

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point mutations, deletions or frameshifts creates a non-functional or truncated hABC1 protein. Individuals suffer from reduced or absent HDL-C and lower serum cholesterol and may exhibit discolored tonsils, enlarged spleen and lymph nodes, corneal clouding and neuropathy due to deposition of cholesteryl esters. In other cholesterol metabolic diseases such as Apo A-I deficiency, LCAT deficiency and Fish Eye disease, HDL-C is also reduced but serum cholesterol levels remain normal (Assmann, G., von Eckardstein, A.. & Brewer, H.B. Jr Familial high density lipoprotein deficiency: Tangier disease. in *The Metabolic and Molecular Basis of Inherited Disease* (eds Scriver, C.R. *et al.*) 2053-2072 (McGraw-Hill, New York, 1995)). Low levels of HDL-C usually indicate a high-risk factor for coronary heart disease. Interestingly, the loss of HDL-C in TD patients does not generally cause predisposition to coronary heart disease.

Tangier disease is exceedingly rare, with only 40 reported families worldwide exhibiting the trait. As such, except for the absence of HDL-C, it is difficult to evaluate which symptoms may be directly attributed to a dysfunctional ABC1 transporter. The murine model of Tangier disease, which is described here, confirms the association of ABC1 with TD and exhibits other phenotypes which have not yet been described in humans. The model may further elucidate why the loss of HDL-C does not directly cause coronary heart disease in TD patients.

The human ATP-binding cassette transporter 1 (hABC1), a member of the ABC superfamily, has recently been cloned and analyzed. Frame shift mutations and single base-pair deletions resulting in truncation of hABC1 have been described and linked to familial HDL deficiency (familial hypoalphalipoproteinemia or FHA) and Tangier disease (TD). Both diseases

are characterized by the lowering or lack of HDL-C. Low serum cholesterol, splenomegaly, enlarged lymph nodes and the deposition of cholesteryl esters in the reticuloendothelial system are also associated with TD.

The murine ABC1 knockout phenotype corroborates the human TD linkage to ABC1. HDL-C is virtually absent in ABC1 knockout mice accompanied by a reduction in serum cholesterol. In addition to these findings, the placenta of ABC1 knockout mice is often grossly malformed, resulting in severe growth retardation, fetal loss and neonatal death. By six months of age, knockout animals begin to develop membranoproliferative glomerulonephritis due to deposition of immunocomplexes containing both immunoglobulin and complement components. They also develop cardiomegaly with ventricular dilation and hypertrophy and ultimately succumb to congestive heart failure. Tangier disease is exceedingly rare and therefore difficult to study. The creation of a knockout model of TD would be very useful in the study of lipid metabolism and cardiovascular disease. Furthermore, a TD mouse model will clarify the role ABC1 normally plays by permitting exploration of the molecular basis of the disease and revealing previously unsuspected phenotypes.

Human TD has not previously been described as causing developmental problems or defective morphogenesis of the placenta. High level expression of ABC1 mRNA in the placenta, however, has been noted in humans. It is not surprising then, to discover in the murine model of TD that major structural defects exist in the placenta. This supports the purported role of ABC1 in cholesterol trafficking since cholesterol is the starting material for steroid hormones and the placenta is a major site of steroidogenesis. Alterations in cholesterol metabolism due to ABC1 loss may therefore result in the defective formation of the placenta. The inventors contemplate that there may be changes in the level of estrogen, testosterone and progesterone detected during the

pregnancy of ABC1 knockout females when compared to pregnant wild-type females. The same alteration in cholesterol metabolism may also result in a reduction in female fertility by altering steroid production during oocyte maturation.

Deposition of cholesteryl esters usually occurs in the reticuloendothelium system in human TD. Previous research with murine macrophages indicates a possible dysfunction in phagocytosis associated with lack of ABC1 function (Luciani, M.F. & Chimini, G. The ATP binding cassette transporter ABC1 is required for the engulfment of corpses generated by apoptotic cell death. *EMBO J.* 15, 226-235 (1996)). We postulate that ABC1 knockout macrophages may be incapable of phagocytosing immune complexes that form naturally in the blood. Serum sickness or autoimmune disease, both of which cause an immense build-up of immune complexes in the blood, will frequently provoke an inflammatory reaction in the kidney glomeruli, due to immunoglobulin deposition. Failure to phagocytose immune complexes would result in a similar build-up of immune complexes in the blood and deposition of immune complex plaques in the kidney glomeruli. The dilated cardiomyopathy may result from the alterations in kidney function and a failure of the normal renal homeostatic mechanisms that control blood pressure. However, the presence of vasculitis in the vessels of the heart suggests that the loss of ABC1 may lead to a general dysregulation of immune complex formation and/or clearance resulting in a generalized immune complex deposition in multiple organs. The finding that normal macrophage function is affected in both mouse and man with differing outcomes may indicate a relationship between cholesterol metabolism and macrophage phagocytosis.

Loss of ABC1 function results in loss of HDL-C and lowered serum cholesterol. This clearly demonstrates the link between Tangier disease, FHA and dysfunctional ABC1 in humans and establishes that ABC1 plays a role in cholesterol metabolism. Furthermore, the lowered serum cholesterol distinguishes the ABC1 knockout as a model of Tangier disease, distinct from the other cholesterol metabolic diseases. The murine model of Tangier disease further demonstrates that loss of ABC1 function may be more detrimental than previously suspected. The marked female infertility and placental malformation may indicate severe developmental consequences in humans if normal ABC1 function is affected. Finally, the renal failure due to immune complex deposition in knockout animals may indicate a previously unsuspected risk factor among patients with FHA or TD, namely the development of an "autoimmune" mediated disease. Further study of the ABC1 knockout will allow better dissection of lipid metabolism and its connection with early development and the immune system.

The following Examples are presented for the purpose of illustrating the present invention and are not to be construed as a limitation on the scope of this invention.

EXAMPLE 1

Gene targeting

Mouse ABC1 clones were isolated from a 129/Ola mouse genomic library. The mouse ABC1 gene contains 48 exons (GenBank accession X75926). The knockout construct was composed of 4 parts arranged in a 5' to 3' order, as illustrated in Figure 1A: (1) A 5 kb DNA fragment from a 129/Ola mouse genomic clone covering exon 18 of the *abc1* gene as the long homologous region for recombination. (2) A 1.2 kb DNA cassette containing a neomycin resistant gene with its own promoter and polyadenylation signal that

replaced a 1.9 kb DNA region containing exons 19, 20, and part of 21. (3) A 0.8kb DNA fragment covering part of exon 21 and 22 of the *abc1* gene. (4) An HSV-thymidine kinase DNA cassette with its own promoter and polyadenylation signal. The *abc1* gene, the neomycin resistant gene and the thymidine kinase gene were in the same orientation of transcription.

In this knockout construct, the mouse *abc1* gene was disrupted by deleting exons 19, 20 and a portion of exon 21, which encode most of the first ATP binding cassette of the ABC1 protein.

Transfection of ES cells with the abc1 DNA construct

Embryonic stem (ES) cells E14 (Hooper et al., 1987, HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 326, 292-295) were maintained at an undifferentiated stage by co-culturing with embryonic fibroblasts (EF) and in culture medium DMEM (15% FCS, 1 mM sodium pyruvate, 0.1 mM b-mercaptoethanol, 2 mM L-glutamine, 100 U penicillin and 100 U streptomycin) containing 1000 U/ml leukemia inhibitory factor (LIF) (Gibco). EF cells were primary fibroblast cultures prepared from day 15-17 mouse fetuses according to the method described by Robertson (Robertson, E.J. (1987) Embryo-derived Stem Cell Lines. In: Teratocarcinomas and Embryonic Stem Cells. E.J. Robertson, ed. (Oxford, Washington DC: IRL Press), p 71-112.). EF cells were treated with 10 mg/ml mitomycin C (Sigma) in culture medium for 2 hours to stop cell division prior to their use as feeder cells.

For DNA transfection, the DNA construct was linearized by NotI digestion. DNA was then precipitated by 2 volumes of ice cold ethanol at -20°C for 1 hour. Precipitated DNA was pelleted by centrifugation, rinsed once with 0.5 ml 70% ethanol, air dried and then dissolved at 1 mg/ml in phosphate-

buffered saline (Gibco). ES cells were harvested by trypsin treatment and resuspended at 6.25 x 10^6 cell/ml in culture medium. DNA construct (20 μg) was added to 0.8 ml of ES cell suspension for electroporation at 250 μF and 340 Volts using the Gene Pulser (BioRad).

Transfected ES cells were plated onto EF coated 90 mm plates at 2.5 x 10^6/plate in culture medium. Two days later, cells were subjected to drug selection in medium containing 400 μg/ml G418 (Geneticin, Gibco) and 0.2 μM ganciclovir. Culture medium was changed daily. Massive cell death was obvious starting day 4 and most of the dead cells were removed through daily medium change. Surviving cell colonies were observable under microscope by day 7 and by day 10 they were visible on the plates without a microscope.

PCR screen of transfected ES cells for homologous recombination

The size of ES colonies on day 11 after transfection was large enough for PCR screening. To collect cell colonies, culture medium in the 90 mm plates was aspirated and 10 ml PBS was added. Individual cell colonies were located with the aid of a stereomicroscope, collected in a 20 ml volume with an autopipetteman and transferred into 96 well-plates. To prepare single cell suspension of the ES colonies, 25 μl of 0.25% trypsin (Gibco) was added per well in 96 well-plates. After 8 minutes of trypsin treatment at 37°C, 25 μl of culture medium was added. All the ES colonies were still maintained in culture as master plates while screening by PCR for homologous recombination events was performed. To prepare master plates, 60 μl of each cell sample was transferred to 96-well plates which had been coated with EF cells and contained 180 μl/well of the culture medium containing G418 and ganciclovir.

For the first round PCR screen, each cell lysate sample was prepared from 12 cell colonies which arrayed as one row of samples in the 96 well-plates.

After the preparation of master plates, the remaining cell samples of about 90 µl/well on every row of the plates were pooled. Cells were pelleted in tubes by centrifugation for 1 minute. After draining all the medium, cells were lysed by adding 30 µl distilled water and brief vortexing. Cell lysates were prepared by first heating at 95°C for 10 minutes, cooling to room temperature and followed by an addition of 1 µl proteinase K (10 mg/ml in water) with brief vortexing, a 90 minute incubation at 50°C for proteinase K digestion, and then 10 minutes at 95°C for heat inactivation of proteinase K.

PCR was carried out using the 9600 GeneAmp system (Perkin Elmer). The reaction mixtures contained 5 µl cell lysate, 4 µM of each of the two oligonucleotide primers, 200 µM each of dATP, dTTP, dCTP, and dGTP, and 5 U AmpliTaq DNA polymerase in PCR buffer (10 mM Tris-Cl, pH8.3, 50 mM KCl,1.5 mM $MgCL_2$ and 0.001% w/v gelatin). The 5' primers ABC3057 [SEQ.ID.NO.:1] 5'-GAGCACATCTGGTTCTATGC-3' and Neo1455 [SEQ.ID.NO.:2] 5'-GCTTCCTCGTGCTTTACGGTAT-3' were specific for the endogenous and altered ABC1 gene, respectively. The 3' primer ABC210R [SEQ.ID.NO.:3] 5'-AAGACACGGTGCTGCTACTGTT-3' was specific for the *abc1* gene in a region flanking the 3' end of the knockout construct . The wild-type *abc1* gene was detected by primers ABC3057 and ABC210R in PCR amplification as a DNA band of 1.3kb. The disrupted *abc1* gene was amplified by primers Neo1455 and ABC210R as a DNA band of 1.0kb. The PCR reaction was 2 cycles of 1 minute at 94°C, 1 minute at 54°C, 2 minutes at 72°C, for 2 cycles; then 40 cycles of 15 seconds at 94°C, forty seconds at 54°C, one minute and thirty seconds at 72°C, and finally a 5 minute elongation period at 72°C.

ES cells in master plates after 3-4 days culture were ready for splitting. Cell colonies in the positive groups were screened individually by a second round of PCR to identify the positive individual colonies. To maintain the

positive groups in culture, cells in the wells were trypsinized by first removing the culture medium, rinsing once with 50 µl PBS, treating with 40 µl 0.25% trypsin for 5 minutes at 37°C, followed by adding 90 µl culture medium. Cells were then resuspended and 20 µl of the cell samples were transferred to master plates which had been coated with EF and filled with 200 µl culture medium containing G418 and ganciclovir. The remaining cells (110 µl/well) were transferred into eppendorf tubes. Cell lysates were prepared and homologous recombination signals were amplified by PCR and detected by hybridization as described in the previous paragraphs.

Confirmation of homologous recombination by genomic Southern hybridization

Homologous recombination was confirmed by Southern hybridization. ES cells derived from the positive colonies in PCR screen were expanded in culture and DNA was extracted as described by Maniatis et al. (Maniatis, T.; Fritsch, E.F.; Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory pp. 280-281). Genomic DNA samples of the putative knockout cell lines were digested with the restriction enzymes EcoRI, separated by 1% agarose gel electrophoresis, blotted onto Hybond-N+ nylon membranes (Amersham) and hybridized with a 253 bp DNA probe specific for the mouse *abc1* gene that was 3' to the knockout region. This DNA probe fragment was obtained by PCR using the *abc1* gene- specific oligonucleotides ABC547 [SEQ.ID.NO.4:] (5'-CCTACCTGCTGCCTTAAATCT-3') and ABC800R [SEQ.ID.NO.5:] (5'-ATGCTACAGTTCCTACAAGC-3'). This probe hybridizes to a 2.2kb band from EcoRI digested ABC1 gene and to a 2.6kb band from a knockout ABC1 gene. As seen in Figure 1C, the wild-type animals demonstrate a single 2.2kb band, while heterozygotic animals demonstrate two bands, 2.2kb and 2.6kb. This DNA probe did not hybridize to the DNA constructs that were integrated randomly in the chromosome.

Generation of chimeric mice by embryo injection

Mouse embryos at 3.5 day gestation stage were collected from the uteri of super-ovulated C57BL/6J mice. About 10-15 ES cells were injected into the blastocoel cavity of the embryos. Injected embryos were transferred into the uteri of pseudo-pregnant CD1 mice at 2.5 day gestation. Mice developed from these embryos were born 17 days later. Since the ES cells used were derived from the 129 Ola mouse strain with the dominant agouti coat color genes, chimeric mice were identified by the agouti coat color from ES derived cells, versus the black color from C57BL/6J mouse embryos.

ES germline mice obtained by chimeric mouse breeding

Chimeric mice were bred with C57BL/6J mice. These crosses are performed to test for the germline transmission of ES cells. Some of the progeny from the breeding are expected to be agouti if the chimeric male had germ line cells derived from ES cells which carry the dominant agouti coat color genes. The disrupted *abc1* gene in mice was detected by genomic hybridization as described in the previous section. Genomic DNA is purified from about 1 cm of tail from each agouti mouse after weaning. The genomic DNA is isolated as described (Laird et al., supra), followed by phenol and phenol:chloroform extractions and ethanol precipitation. Genomic DNAs are digested with EcoRI, and hybridized with the 3' flanking DNA specific for the *abc1* gene as described earlier.

Generation of homozygous knockout mice from breeding of heterozygous knockout mice

Female heterozygous knockout mice were mated with male heterozygous knockout littermates. It is expected that half of the pups are heterozygous for the disrupted gene, one-quarter carry only the disrupted gene

and one quarter carry only the wild-type gene. Surviving offspring were genotyped by Southern hybridization as described above. Continued breeding of homozygous knockout mice were maintained by crossing heterozygous females with knockout males. As shown in Figure 1B, mice that were wild-type, heterozygous and homozygous for the disrupted *abc1* gene were identified by the PCR method described in the previous paragraph.

EXAMPLE 2

Characterization of ABC1 Knockout Mice and Cells Derived From the Mice

Lipid analysis

Cholesterol and HDL-C from six mice of each genotype were analyzed by standard enzymatic means on an Olympus AU520 mulitchannel analyser (Quest Diagnostics, San Diego, CA).

HDL loss is the direct result of ABC1 dysfunction.

The primary hallmark of Tangier disease is the lack of HDL-C and lowered serum cholesterol, often accompanied by neuropathy, discoloration of the tonsils, clouding of the cornea and enlargement of the spleen and lymph nodes due to deposition of cholesteryl esters (Assmann, G., von Eckardstein, A.. & Brewer, H.B. Jr Familial high density lipoprotein deficiency: Tangier disease. in *The Metabolic and Molecular Basis of Inherited Disease* (eds Scriver, C.R. *et al.*) 2053-2072 (McGraw-Hill, New York, 1995)). Assessment of the physiological consequences of a non-functioning ABC1 transporter is difficult due to the limited number of TD cases. FHA is a more common disease, but apparently is caused by multiple factors. Functional loss of ABC1 in a murine model clearly demonstrates that the major characteristic of TD, lack of HDL-C, is present and substantiates the link between mutated ABC1 and TD. Like TD patients, HDL-C levels in ABC1 knockout mice are almost non-existent while in heterozygous mice HDL-C levels are roughly one-half of the levels found in

normal mice. Cholesterol levels are also significantly reduced in a similar manner (Table I).

Table I. HDL-C and Cholesterol Levels in ABC1 Knockout Mice are Significantly Reduced

Genotype	HDL-C(mg/dL)	Cholesterol (mg/dL)
Wild-Type	77.3±6.1	100.7±6.2
Heterozygous	41.0±8.9	60.2±13
Homozygous	2.3±0.5	22.6±9.8

Few of the other symptoms associated with TD in human patients have been noted in *ABC1* knockout mice. Splenomegaly and minor swelling of the lymph nodes with finely lined yellow-white traceries radiating outwards has occasionally been noted. Taken together, this data indicates that the murine version of Tangier disease is identical to the human disease with regard to HDL-C loss and lowering of serum cholesterol.

In-situ Hybridization

Tissue sections were mounted onto poly-L-lysine coated slides and fixed an additional 30 min in 10% formalin, digested with proteinase K (10 µg/ml in 50 mM Tris HCl, pH 7.5, and 5 mM EDTA) for 30 min, at 37°C, followed by acetylation (0.25% acetic anhydride in 0.1M triethanolamine, pH 8.0) and dehydration by alcohol. Each slide received 100 µl of hybridization mixture, containing the ^{35}S-UTP labeled probe LM243 (10^7 cpm/ml). The slides were coverslipped and incubated at 60°C in an oven overnight.

After the incorporation of labeled probe overnight, the tissue was treated with 20 µg/ml of ribonuclease A for 30 min, at 37°C, washed for 30 min in 15 mM NaCl/1.5 mM sodium citrate (SSC) at 70°C, dehydrated with ethanol and exposed to X-ray films for 6 days. The slides were then defatted in xylene, rinsed in ethanol, dried, dipped in NBT2 nuclear emulsion (Eastman Kodak,

Rochester, NY, diluted 1:1 with distilled water), and stored in the dark at 4°C. Following 22 days, slides were developed in D-19 (Eastman Kodak) for 3.5 min at 14°C, rinsed in distilled water, fixed with Polymax T Fixer (Eastman Kodak) for 4 min at 14°C, and washed in distilled water for 1 h. We defined positively labeled cells as any accumulation of silver grains within a cell-sized area that was five times above background or control levels. Antisense and sense (control) cRNA probes for the ABC1 were generated from the cDNAs. Labeled antisense and sense probes for the ligand were synthesized following linearization with HindIII or EcoRI, using T3 or T7, respectively. The cDNA was purified with Qiagen (Santa Clarita, CA) following the linearization. Probes were labeled with ^{35}S-UTP. Unincorporated nucleotides were removed by G-50 Sephadex Quick Spin columns (Boehringer Mannheim, Mannheim, Germany). The labeled sense strands served as controls and did not show any specific labeling of cellular localization. All restriction enzymes and RNA polymerases were obtained from Boehringer Mannheim. Reference for In-Situ: Simmons, D.M., Arriza, J.L. & Swanson, L.W. A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radiolabeled single-stranded RNA probes. *J. Histotechnol.* 12, 169-181(1989).

Loss of ABC1 results in severe placental malformation and oxidative distress. Other phenotypic traits that could be characteristic of TD may escape detection because the disease is so rare, especially if they are fatal at an extremely early stage of development. The highest level of ABC1 mRNA expression in humans is in the placenta (Luciani, M.F. & Chimini, G. The ATP binding cassette transporter ABC1 is required for the engulfment of corpses generated by apoptotic cell death. *EMBO J.* 15, 226-235 (1996)). In mice, ABC1 mRNA expression is clearly seen lining a decidual maternal blood vessel of the placenta and is present throughout the trophoblast (Fig. 2). Placenta derived from an ABC1 knockout fetus from either the 55.2 or 64.7 lines shows various

degrees of defects ranging from an almost normal structure to a badly distorted, functionally impaired tissue. Placenta from *ABC1* heterozygote fetus also appears slightly abnormal. The structure of the labyrinthine trophoblasts is the most malformed.

A well-formed labyrinth is comprised of a lacy, open structure with a consistent symmetry. In most knockout placenta the labyrinth is much more cellular and the symmetry is distorted (Fig. 3A). Hemorrhages, cell debris and ragged inclusions of the spongiotrophoblast are prevalent (Fig. 3B). These defects may compromise oxygen exchange between mother and fetus. In homozygous crosses, intrauterine growth retardation is evident, accompanied by fetal death and resorption *in utero* as early as day 14 p.c. (Fig. 3C). As pregnancy progresses, fetal distress becomes even more apparent. At day 19 p.c. the amniotic sacs are stained brown, consistent with the release of meconium into the amniotic fluid (Fig. 3D). Often half of the remaining pups are dead *in utero*, as measured by lack of reflexive movement. Pups that survive to be born often succumb in the first 24-48 hours after birth. Autopsy indicates the lungs are congested with blood and are extremely cellular (Fig. 3E). The histology is reminiscent of bronchopulmonary dysplasia, the sequelae of hyaline membrane disease. It may also indicate inhalation of meconium-tainted amniotic fluid *in utero*. Finally, a handful of neonates runt as early as five days after birth and die from similar causes by 2.5 weeks.Heterozygous crosses of ABC1 mice results only an 8% survival rate of knockouts (176 animals analyzed – Table II). This survival rate is increased to 28% in heterozygote/homozygote crosses (76 animals analyzed). Interestingly, a marked sex bias against male homozygotes is present. Only one-quarter of the male homozygotes survive (6.5%) compared to the expected Mendelian ration for male homozygotes (25%). Lastly, only 10% of female homozygotes ever succeed in bearing 1-2 viable offspring. Thus, loss of functional ABC1 consequently results in a severe developmental

defect of the placenta, leading to intrauterine growth retardation and neonatal death and markedly reduces female fertility.

TableII. Mendelian Ratios of ABC1 Mating are Biased Against Knockout Mice

Cross (male x female)	Genotype		
	Wild Type	Heterozygote	Homozygote
Heterozygote x Heterozygote	34%	56%	8%
Homozygote x Homozygote	-	72%	28%

Loss of ABC1 results in immune complex deposition in kidney glomeruli and congestive heart failure.

ABC1 knockout mice that survive to be weaned appear to develop normally and mature into apparently healthy adults. Between 4-6 months of age the mice of both lines begin to develop respiratory distress and shed granular casts into their urine. Necropsy examination reveals lungs heavily filled with blood and cardiomegaly with dilated hypertrophied left and right ventricles. There is occasional evidence of vasculitis around the cardiac vessels. The kidneys are pale tan in color. Microscopic examination reveals boxcar nuclei in the heart consistent with cardiac hypertrophy with frank pulmonary hemorrhages as well as severe congestion of the lungs, liver and spleen, and scarred kidney glomeruli. The glomeruli show evidence of inflammatory infiltrates, thickened and "split" glomerular basement membranes and proliferation of mesangial cells. Scarring of glomeruli was visible when sections were stained with trichrome (Fig. 4A). Immunohistochemistry confirms the deposition of both Ig and C3 complement components in the glomeruli characteristic of membranoproliferative glomerulonephritis type I (Fig. 4B). To summarize,in knockout ABC1 mice we find evidence of immune complex and complement

deposition in kidney glomeruli, inflammation, glomeruli nephritis, cardiomegaly and congestive heart failure.

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12. Assmann, G., von Eckardstein, A.. & Brewer, H.B. Jr Familial high density lipoprotein deficiency: Tangier disease. in *The Metabolic and Molecular Basis of Inherited Disease* (eds Scriver, C.R. *et al.*) 2053-2072 (McGraw-Hill, New York, 1995).

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15. Simmons, D.M., Arriza, J.L. & Swanson, L.W. A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radiolabeled single-stranded RNA probes. *J. Histotechnol.* 12, 169-181(1989).

WHAT IS CLAIMED IS:

1. A transgenic mouse whose somatic and germ cells contain a gene encoding a non-functional *abc1* gene, the non-functional *abc1* gene having been targeted to replace a wild-type *abc1* gene into the mouse or an ancestor of the mouse at an embryonic stage using embryonic stem cells, wherein said mouse has reduced serum cholesterol compared to a wild type *abc1*mouse.

2. The mouse of Claim 1, wherein said mouse is fertile and capable of transmitting the non-functional *abc1* gene to its offspring.

3. The mouse of Claim 1, wherein the non-functional *abc1* gene has been introduced into an ancestor of the mouse at an embryonic stage by microinjection of embryonic stem cells into mouse blastocysts.

4. The mouse of Claim 1, wherein the non-functional *abc1* gene has been introduced into the mouse at an embryonic stage by microinjection of embryonic stem cells into mouse blastocysts.

5. A method of producing a mouse whose somatic and germ cells contain a non-functional *abc1* gene, the non-functional *abc1* gene having been targeted to replace a wild-type *abc1* gene in the mouse or an ancestor of the mouse at an embryonic stage using embryonic stem cells, which comprises:

(a) introducing a non-functional *abc1* gene designed to target the wild type *abc1* gene into mouse embryonic stem cells;

(b) inserting the embryonic stem cells containing the non-functional *abc1* gene into mouse blastocysts;

(c) transplanting the injected blastocysts into a recipient mouse; and

(d) allowing the embryo to develop producing a founder transgenic mouse, wherein said mouse has reduced serum cholesterol compared to a wild type *abc1* mouse.

6. The method of Claim 5 wherein the introducing of step (a) is by electroporation or microinjection.

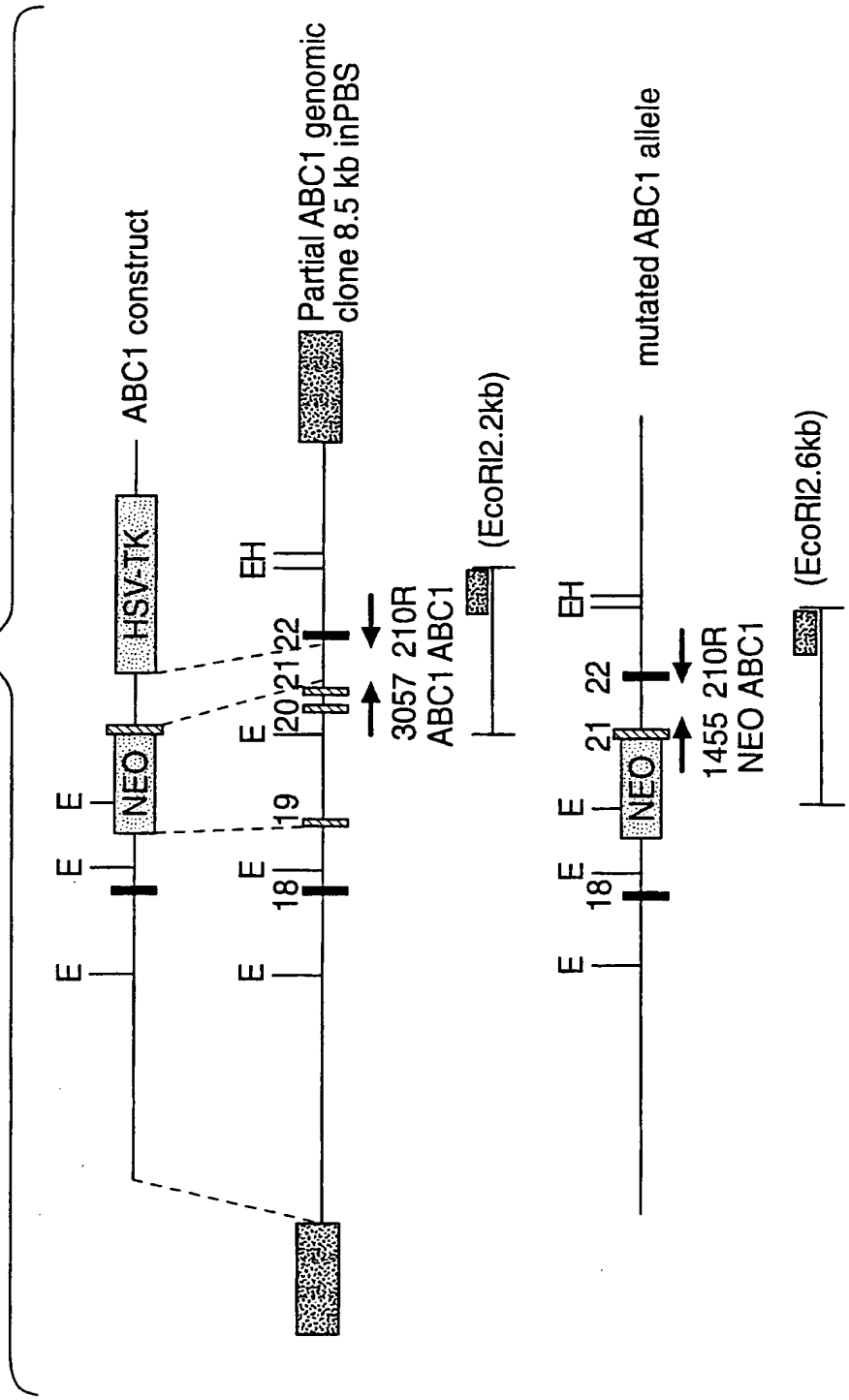
7. The method of Claim 5 which further comprises the steps:

(e) breeding the transgenic mice with wild-type mice to obtain heterozygous (F1) mice; and

(f) breeding the heterozygous (F1) mice to generate homozygous (F2) ABC1 deficient transgenic mice.

8. A cell line derived from the transgenic mouse of Claim 1.

FIG. 1A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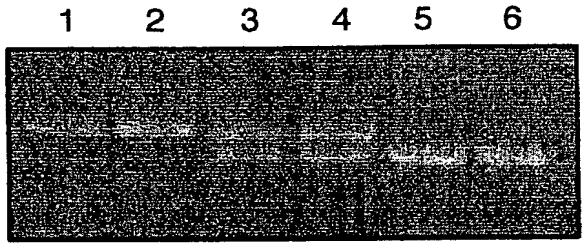

FIG. 1C

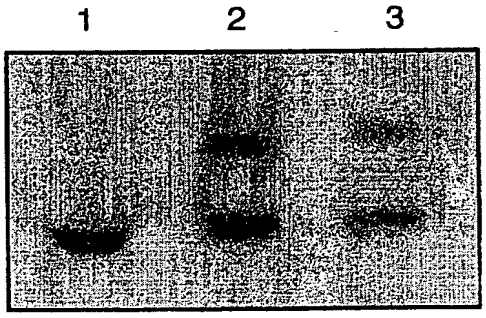

FIG. 2

FIG. 3A

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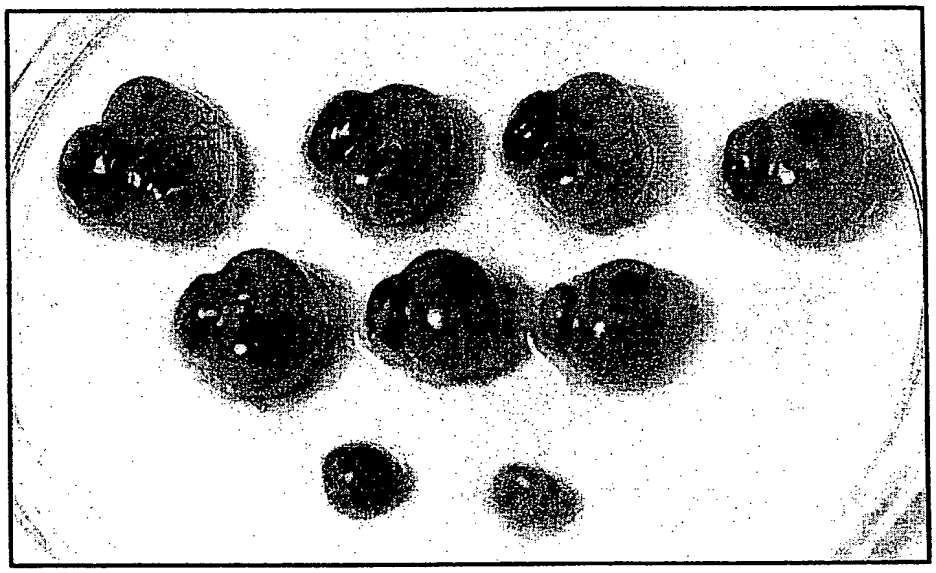
WILDTYPE MOUSE

FIG. 3B

ABC1 TRANSGENIC MOUSE

WILDTYPE MOUSE

FIG. 3C



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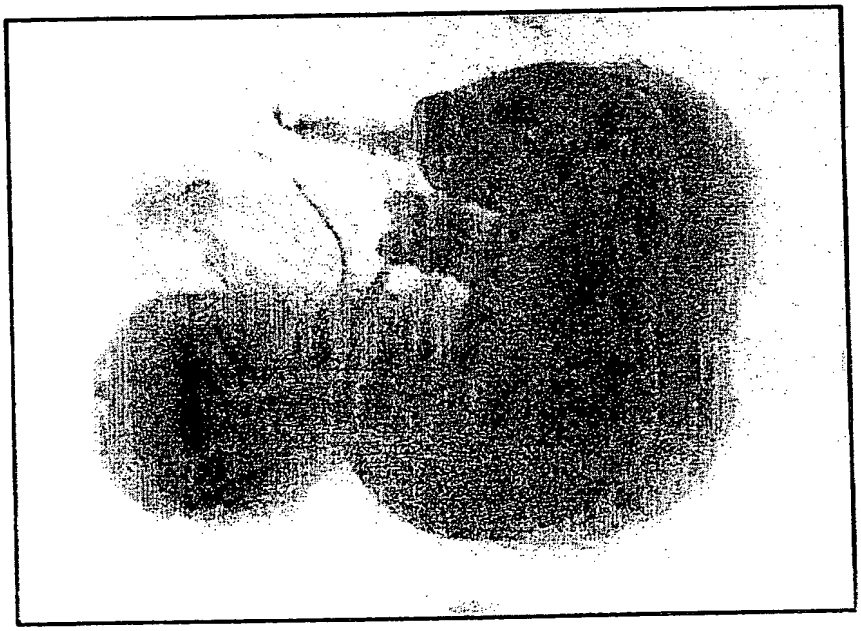

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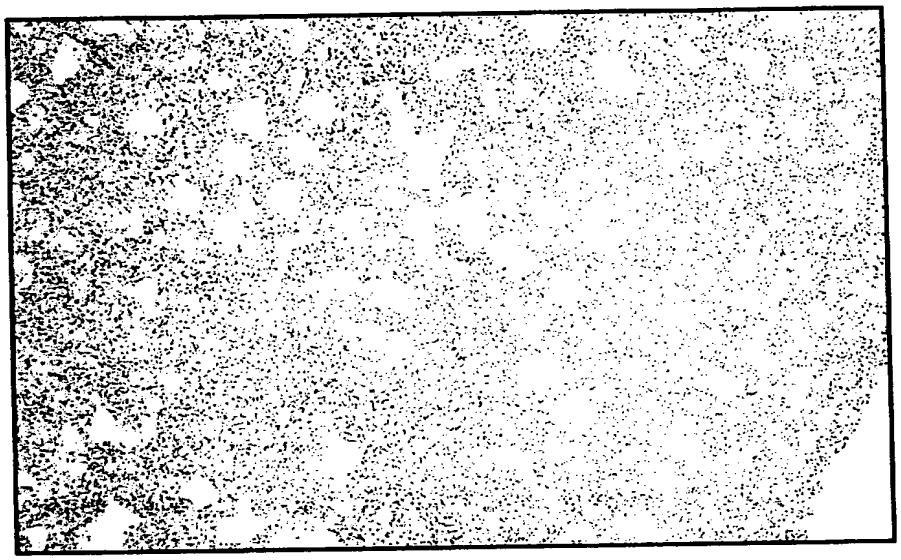

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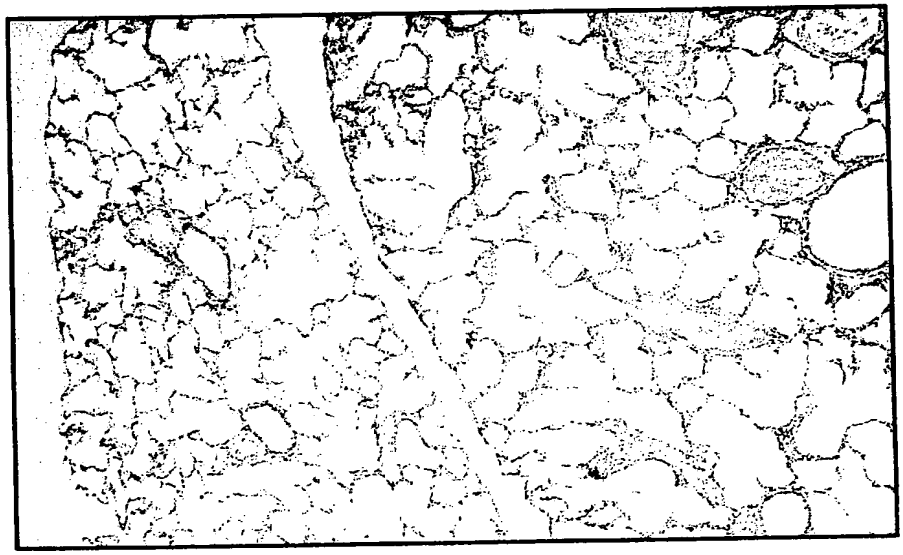

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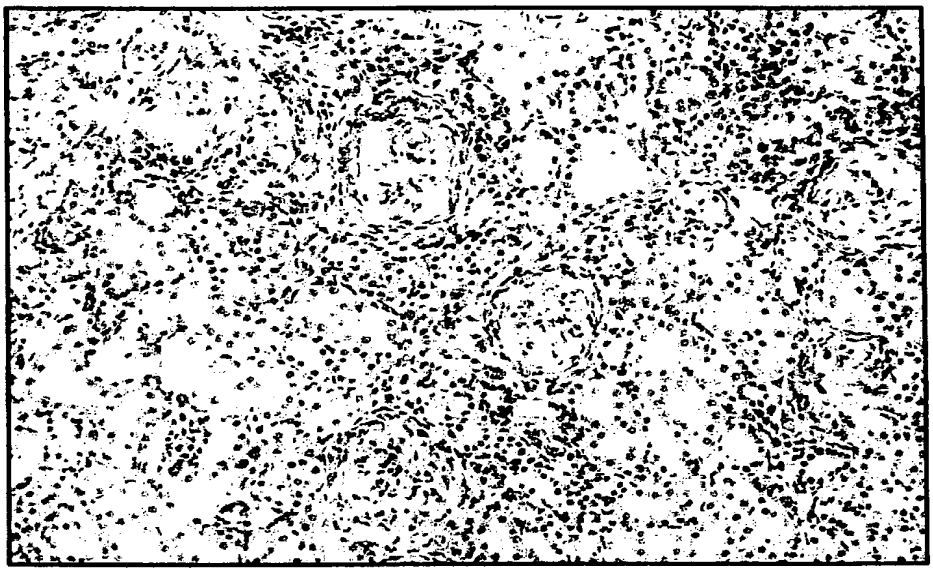

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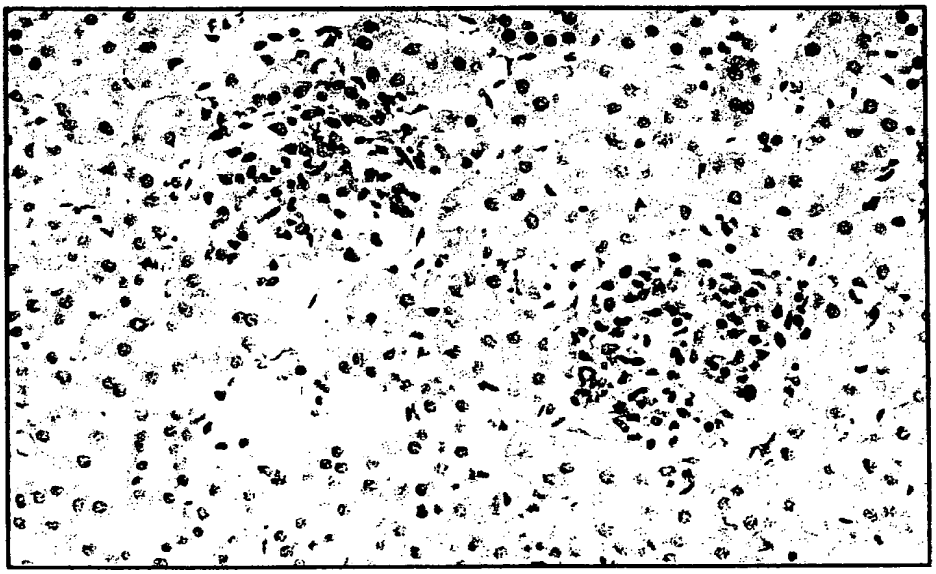

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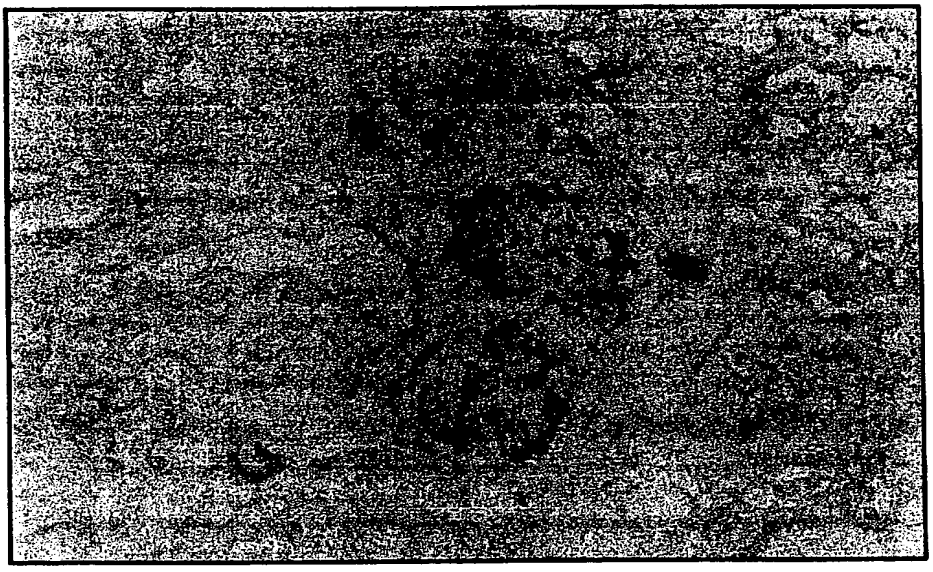

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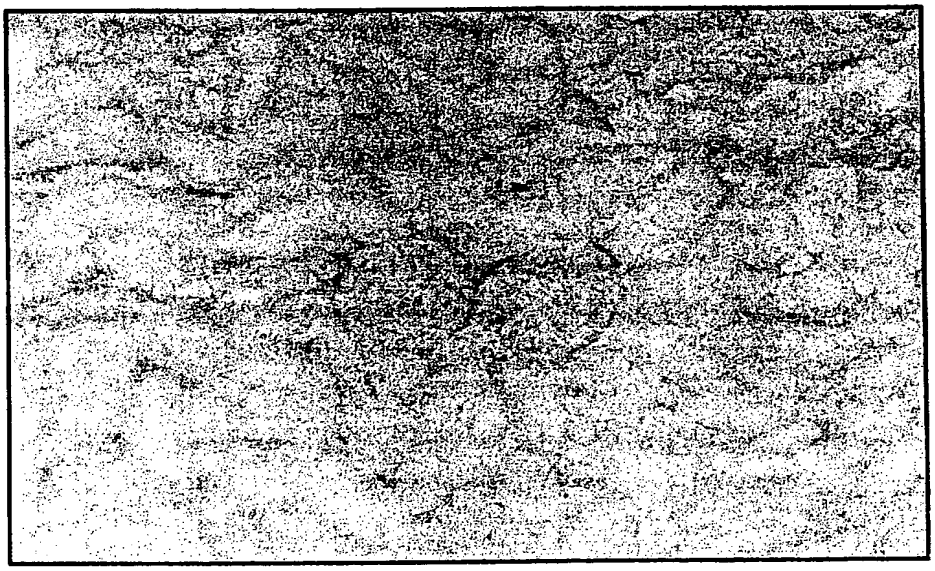

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Voland, Joseph R

Peterson, Per A

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27742

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01K 67/00, 67/027; C12N 5/00, 5/10, 15/00
US CL : 800/8, 9, 13, 18, 21, 25; 435/325, 354

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/8, 9, 13, 18, 21, 25; 435/325, 354

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ----- Y, P	ORSO, E. et al. Transport of lipids from Golgi to plasma membrane is defective in Tangier disease patients and Abc1-deficient mice. Nature Genet. 01 February 2000, Vol. 24, No. 2, pages 192-196, see the entire document, especially page 192.	1-2 ----- 3-8
X, P ---- Y, P	MCNEISH, J. et al. High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. Proc. Natl. Acad. Sci. U.S.A. 11 April 2000, Vol. 97, No. 8, pages 4245-4250, see entire document, especially Materials and Methods section.	1-7 ----- 8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 04 NOVEMBER 2000	Date of mailing of the international search report 28 DEC 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer QUANG NGUYEN, PH.D. Telephone No. (703) 308-0198

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27742

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LUCIANI, M.F. et al. Cloning of two novel ABC transporters mapping on human chromosome 9. Genomics. 01 May 1994, Vol. 21, pages 150-159, see the abstract.	1-8
Y	WENG, J. et al. Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice. Cell. 09 July 1999, Vol. 98, pages 13-23, see column 2, page 20.	1-8
Y	YOUNG, S.G. et al. The ABCs of cholesterol efflux. Nature Genet. 01 August 1999, Vol. 22, pages 316-318, see the entire document.	1-8

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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG, MEDLINE, EMBASE, BIOSIS, APS
Search terms: abc1, abc?, transgenic mouse, knockout mouse, abc1 deficient mouse, cell or cell line.